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The interaction between casein kinase I α and 14-3-3 is phosphorylation dependent

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Keywords

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We have previously shown that casein kinase (CK) I α from mammalian brain phosphorylates 14-3-3 ζ and τ isoforms on residue 233. In the present study, we show that CKI α associates with 14-3-3 both *in vitro* and *in vivo*. The interaction between CKI α and 14-3-3 is dependent on CKI α phosphorylation, unlike centaurin- α 1 (also known as ADAP1), which binds to unphosphorylated CKI α on the same region. CKI α preferentially interacts with mammalian η and γ 14-3-3 isoforms, and peptides that bind to the 14-3-3 binding pocket prevent this interaction. The region containing Ser218 in this CKI α binding site was mutated and the interaction between CKI α and 14-3-3 was reduced. We subsequently identified a second phosphorylation-dependent 14-3-3 binding site within CKI α containing Ser242 that may be the principal site of interaction. We also show that both fission and budding yeast CKI kinase homologues phosphorylate mammalian and budding yeast (BMH1 and BMH2) 14-3-3 at the equivalent site.

Structured digital abstract

- A list of the large number of protein-protein interactions described in this article is available via the MINT article ID [MINT-7264069](https://www.ebi.ac.uk/Tools/mint/MINT-7264069)

Introduction

The 14-3-3 family is highly conserved over a wide range of mammalian species, where the individual isoforms (β , γ , ϵ , ζ , η , σ and τ) are either identical or contain a few conservative substitutions [1]. Homologues of 14-3-3 proteins have also been found in a broad range of eukaryotes [2,3]. Almost every known organism expresses multiple 14-3-3 isoforms [4]. 14-3-3 modulates interactions between proteins involved in the regulation of the cell cycle, intracellular trafficking/targeting, signal transduction, cytoskeletal struc-

ture and transcription. The regulatory roles for 14-3-3 isoforms include nuclear trafficking as well as the direct interaction with cruciform DNA (i.e. involved in transcription regulation) and with a number of receptors, small G-proteins and their regulators. In many cases, these proteins show a distinct preference for a particular isoform(s) of 14-3-3 [1]. A specific repertoire of 14-3-3 dimers may influence which interacting proteins could be brought together. We have demonstrated the preference for both mammalian and yeast

Abbreviations

CK, casein kinase; db-cAMP, dibutyryl-cAMP; GST, glutathione S-transferase; HEK, human embryo kidney; IVTT, *in vitro* transcription translation; PKA, protein kinase A; PKC, protein kinase C; Ppase, phosphatase.

14-3-3 isoforms to dimerize with specific partners *in vivo* [5]. Interaction is most often regulated by phosphorylation of the interacting protein and/or the 14-3-3 isoform itself. The structures of 14-3-3 dimers [6–11] including the site of interaction of both phospho- and unphosphorylated motifs are known. Nonphosphorylated binding motifs can also be of high affinity and may show more isoform-dependence in their interaction [12]. Binding of a protein through two distinct binding motifs to a dimeric 14-3-3 may also be essential for full interaction [13].

Budding and fission yeast each have two homologues of 14-3-3 and the deletion of both is normally lethal [14]. Deletion of a single *BMH* gene affects yeast growth and cell division [15], although a particular strain of *Saccharomyces cerevisiae* was found to be viable with a double deletion of *BMH1* and *BMH2* [16]. The strain is, however, defective in rat sarcoma/mitogen-activated protein kinase cascade signalling during pseudohyphal development.

The mammalian 14-3-3 isoforms β and ζ can be phosphorylated *in vivo* on Ser185 [17] and, interestingly, Ser185 is located in the tertiary structure adjacent to residue 233 [1]. Tsuruta *et al.* [18] have shown that activated c-Jun N-terminal kinase promotes Bax translocation to mitochondria through phosphorylation of 14-3-3 σ and ζ at sites equivalent to Ser185, which led to the dissociation of Bax. The expression of phosphorylation-defective mutants of 14-3-3 blocked c-Jun N-terminal kinase-induced Bax translocation to mitochondria, cytochrome *c* release and apoptosis [19].

Members of the casein kinase (CK) I family have diverse roles, including the regulation of p53; circadian rhythm; Wnt signalling pathway; membrane trafficking; regulation of centrosomes and spindle formation; actin cytoskeleton organization; cell cycle progression; and membrane trafficking and RNA processing, [20,21]. They co-localize in neurones with synaptic vesicle markers and phosphorylate some synaptic vesicle-associated proteins. Seven isoforms from distinct genes are expressed in mammals (CKI α , β , δ , ϵ , $\gamma 1$, $\gamma 2$, $\gamma 3$) and additional CKI forms occur through alternative splicing. CKI β is only found in bovine brain and may be the bovine equivalent of the CKI $\alpha 2$ splice variant.

We identified CKI α as the brain kinase that phosphorylated 14-3-3 ζ on Thr233 [22]. 14-3-3 τ and yeast 14-3-3 (*BMH1* and *BMH2*) were also phosphorylated on the equivalent sites [23]. *In vivo* phosphorylation of 14-3-3 ζ at this site negatively regulates its binding to c-Raf, and may be important in Raf-mediated signal transduction [24]. We subsequently confirmed the interaction of a number of proteins that co-purified with

CKI α in brain by co-immunoprecipitation and affinity chromatography [25–27]. These included centaurin- $\alpha 1$, comprising the phosphatidylinositol 3,4,5-triphosphate-binding protein that associates with presynaptic vesicular structures [28]. CKI α colocalizes in neurones with synaptic vesicle markers and phosphorylates some synaptic vesicle-associated proteins [29].

We subsequently identified the site of interaction of CKI α with centaurin- $\alpha 1$ in a loop region contained within the kinase domain comprising residues 217–233 [26]. The original MS search that identified CKI α from the co-purifying protein complex included the tryptic peptide containing Ser218. However, the data clearly showed no indication of phosphorylation of CKI α on this residue. From crystallographic studies [30], the loop region has been postulated to represent a site of interaction with other proteins. On the basis of this observation, we showed that a nonphosphorylated synthetic peptide corresponding to this region could bind a number of proteins from the brain, including actin, importin- $\alpha 1$, importin- β , protein phosphatase 2Ac, centaurin- $\alpha 1$ and HMG1 [25]. However, 14-3-3 was not identified during those investigations.

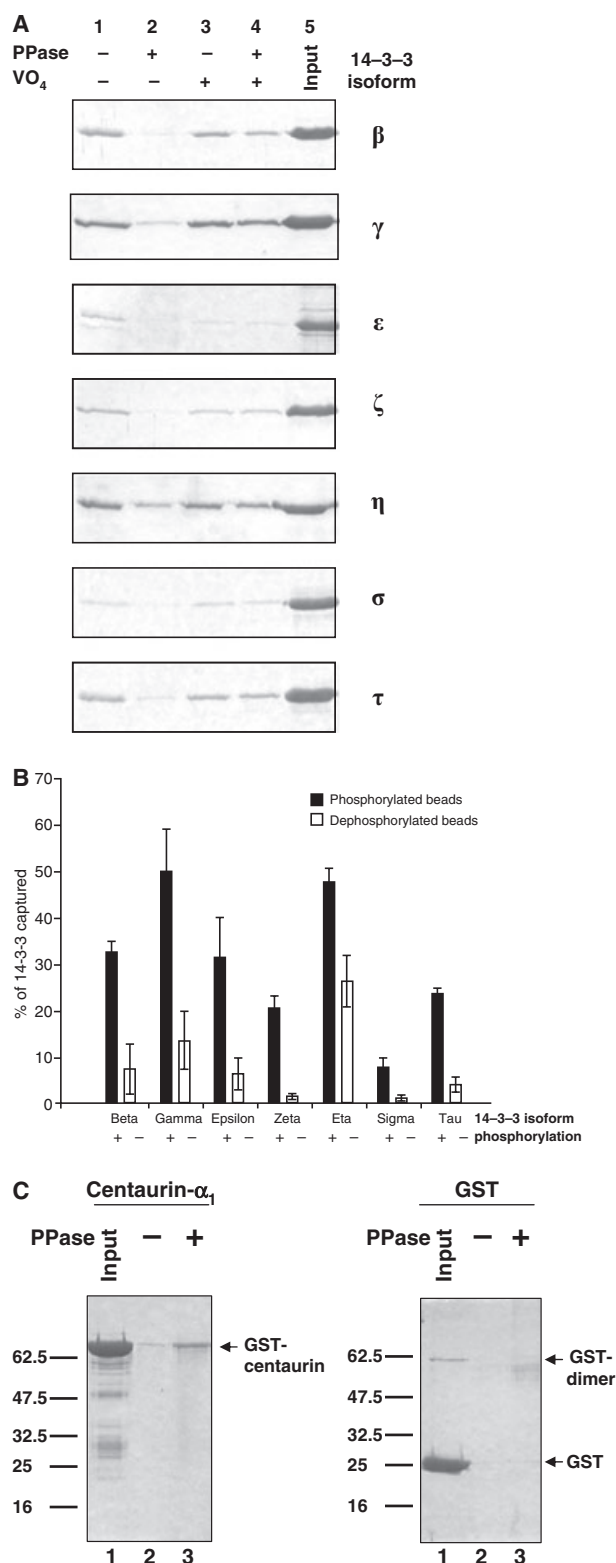
One of the aims of the present study was to examine the possibility that, as well as being a substrate of CKI α , 14-3-3 could form a stable complex with CKI. We predicted that the interaction could occur within the interaction loop containing residue Ser218 (on the condition that it was phosphorylated) because this would produce a potential 14-3-3 binding motif: RTpS²¹⁸LP. The kinase domain is highly conserved between members of the CKI family, although unique N- and C-terminal tails characterize each isoform. An additional aim of the study was to investigate which CKI homologues might interact with 14-3-3 and, in the present study, we show that CKI associates with 14-3-3 both *in vitro* and *in vivo*.

Results

Phosphorylation-dependent interaction between 14-3-3 and CKI α

To investigate whether the region 214–226 representing the proposed ‘interaction loop’ of CKI α could bind 14-3-3, a peptide (C-FNRTpSLPWQGLKA, where pS is phosphoserine) corresponding to this region was coupled to Sulfolink affinity beads.

Equal amounts of the phospho-CKI α peptide were shown to bind to all 14-3-3 isoforms but preferentially with η and γ 14-3-3 isoforms (which have relatively high sequence similarity) [1] and, to a lesser extent, to 14-3-3 σ and ϵ isoforms (Fig. 1A). Dephosphorylation



of the peptide, by lambda phosphatase (PPase) treatment resulted in a loss of interaction with all isoforms (Fig. 1A, lanes 2). Control experiments after the incu-

Fig. 1. A phosphopeptide corresponding to residues 213–226 of CKI α associates with all 14-3-3 isoforms in a phospho-dependant manner. (A) Sulfolink beads conjugated to ~ 20 μ g of peptide corresponding to residues 214–226 (C-FNRTpSLPWQGLKA) of CKI α were incubated with all 14-3-3 isoforms (panels 1–5), washed three times and subjected to SDS-PAGE followed by Coomassie Brilliant Blue staining. Lane 1, untreated beads; lane 2, beads treated with lambda phosphatase (PPase); lane 3, beads incubated with the phosphatase inhibitor sodium orthovanadate (Na₃VO₄); lane 4, control PPase with the inclusion of phosphatase inhibitor (Na₃VO₄) to verify that the enzyme does not interfere with binding of 14-3-3; lane 5, amount of 14-3-3 incubated with the peptide beads (input). (B) IMAGEJ software (<http://rsbweb.nih.gov/ij/>) was used to measure the density of bands corresponding to 14-3-3 and the SD plotted using SIGMAPLOT (Systat Software, Inc., Chicago, IL, USA). The values shown are the percentage of the intensities of 14-3-3 captured by the peptide compared to the intensity of 14-3-3 applied to the beads (input). These results are taken from three independent experiments. (C) An affinity column containing phospho-CKI peptide was prepared as in (A) and the binding of the following constructs was analysed as before. Left panel: GST-centaurin- α_1 ; lanes 1, input (equal to the quantity added to the beads); lanes 2, phosphopeptide affinity column; lane 3, phosphopeptide affinity column after lambda phosphatase treatment. Right panel: control, GST alone at a similar level. These results are typical of three independent experiments.

bation of the PPase with the inhibitor, vanadate (VO₄) (lanes 4) indicated that the interaction is phospho-dependent and that the effect was caused by PPase masking the binding of the peptide to 14-3-3. More of the η and γ 14-3-3 isoforms bound to both the phospho- and dephospho-peptide, with σ binding approximately five-fold less (Fig. 1B). Dephosphorylation of Ser218 reduces the binding to all of the 14-3-3 isoforms. The opposite result was observed with centaurin- α_1 (Fig. 1C), which binds robustly to the dephospho-peptide in contrast to the very low amounts associating with the phospho-peptide. Therefore, the interaction between centaurin- α_1 and CKI α occurs when CKI α is dephosphorylated.

14-3-3 isoforms associate with CKI α both *in vitro* and *in vivo*

To determine the 14-3-3 isoform specificity of the 14-3-3:CKI α interaction, six isoforms of recombinant 14-3-3 were added to lysates from human embryo kidney (HEK) 293 cells transfected with HA-CKI α (Fig. 2). Recombinant glutathione *S*-transferase (GST) (control) and GST-14-3-3 proteins were incubated with the cell lysate, pulled down with glutathione Sepharose and western blotted for CKI α using α -HA antibodies. The results demonstrated that more CKI α

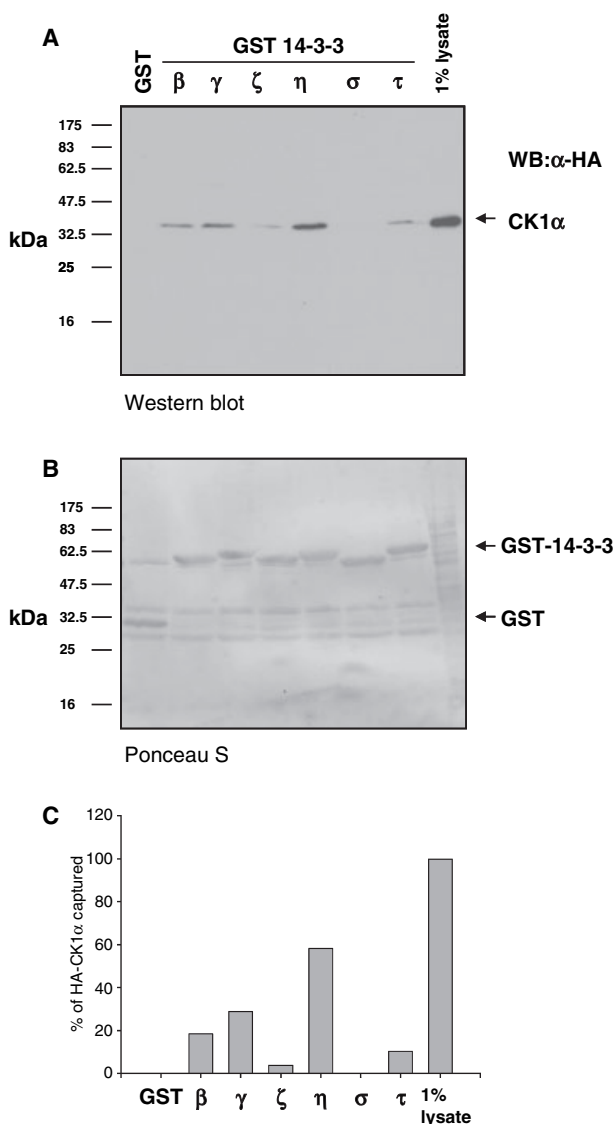


Fig. 2. 14-3-3 isoforms associate with CKI α *in vitro*. (A) COS-1 cells were transfected with HA-CKI α , the lysates were then clarified before the addition of 10 μ g of recombinant GST-14-3-3. Each sample was rotated at 4 °C for 1 h before the addition of glutathione beads. After 2 h, each pull-down was washed three times in lysis buffer before separation by SDS-PAGE. The gel was transferred and western blotted with α -HA. GST control lane is shown in the far left hand lane. In the far right hand lane, 1% of the lysate that was incubated with each 14-3-3 isoform. (B) Ponceau S staining showing equal loading of recombinant 14-3-3 isoforms. (C) Densitometry analysis of the blot in (A), showing the amount of HA-CKI α that binds to each 14-3-3 isoform, plotted as a percentage of the input. This experiment was carried out in duplicate with similar results being obtained.

bound to the 14-3-3 η and γ isoforms (Fig. 2A). Densitometry analysis of the blot was performed to determine the binding levels between the 14-3-3 isoforms.

The amount of CKI α pulled down by equal amounts of recombinant 14-3-3 isoforms (as judged by Ponceau S staining; Fig. 2B) was compared with the amount of CKI α present in 1% of the lysate. It is clear that 14-3-3 η interacts more strongly than the other isoforms, followed by γ , β , τ and ζ . The σ isoform did not interact at all.

To verify the isoform specificity of the 14-3-3:CKI α interaction *in vivo*, a reciprocal experiment was performed, whereby the CKI α binding affinity to the five 14-3-3 isoforms present in abundance in HEK293 cells was screened. This established that, in unstimulated cells, native endogenous 14-3-3 η and γ appear to associate more than the other isoforms (Fig. 3A). Although it is not possible to discern quantitatively the binding affinity for the η and γ isoforms, as a result of the differing titres of the antibodies, there is still a clear difference between the isoforms. To check that similar levels of CKI α were present in each binding assay, a western blot was also performed using α -HA antibodies (Fig. 3B). A control immunoprecipitation is also shown where a non-HA-immune IgG was incubated in the cell lysate.

It is interesting to note that the two isoforms, 14-3-3 η and γ , which were identified in the present study as associating with CKI α to a greater degree *in vitro* and *in vivo*, are the same isoforms identified that bind best to the phospho Ser218 peptide (Fig. 1). Interestingly, these two isoforms have recently been identified as being able to bind calmodulin-dependent protein kinase kinase, in contrast to 14-3-3 ζ and ϵ [31] and, in so doing, protect it from dephosphorylation in HEK293 cells. The high sequence similarity between η and γ 14-3-3 (74% identity) could explain their similar binding characteristics [1,32].

14-3-3 interacts with other mammalian CKI isoforms

To test whether 14-3-3 was able to interact with other CKI isoforms, CKI ϵ was transfected into COS-7 cells and the lysate was pulled down with GST 14-3-3 η and GST as a control (Fig. 4). Western blotting with α -HA antibody showed that CKI ϵ interacted with GST 14-3-3 η , but not the GST control (middle panel). Because this suggests that 14-3-3 may interact with other CKI isoforms if they contain a consensus 14-3-3 motif at the equivalent position of either residue 218 or 242 (Fig. 4B), we therefore extended our analysis to the interactions between the yeast (*S. cerevisiae*) CKI homologue (HRR25) and both mammalian 14-3-3 and yeast 14-3-3 homologues (BMH1 and BMH2).

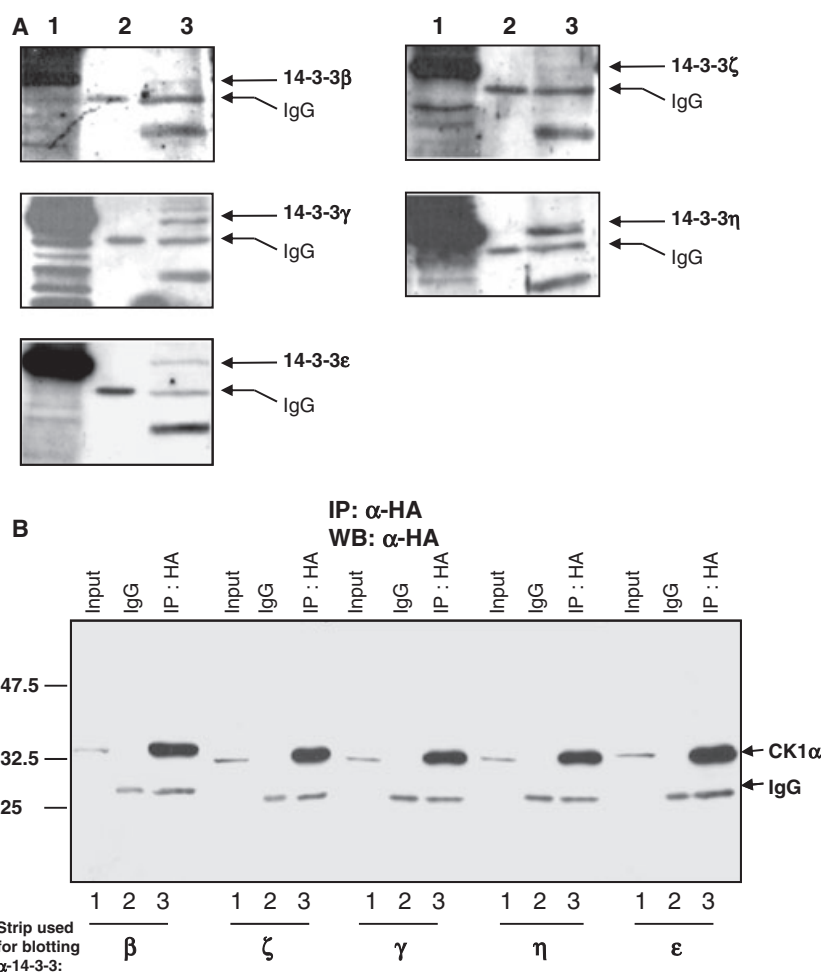


Fig. 3. 14-3-3 isoforms bind CKI α *in vivo*. (A) Unstimulated HEK293 cells were transfected with HA-CKI α and immunoprecipitated with α -HA-conjugated beads. After extensive washing in lysis buffer, the immunoprecipitates were subjected to SDS-PAGE and western blotted with antibodies specific to each 14-3-3 isoform. Left hand lanes show a non-immune IgG control and the right lanes show the α -HA immunoprecipitation. Lane 1, 1% of the lysate in unstimulated HEK293 cells transfected with HA-CKI; lane 2, immunoprecipitation with a non-immune IgG; lane 3, the α -HA immunoprecipitation. (B) Equal amounts of CKI α were present for the assessment of 14-3-3 immunoprecipitations. This shows a re-probe of (A) with α -HA antibody. Lane 1, 1% of the lysate in unstimulated HEK293 cells transfected with HA-CKI; lane 2, α -HA-conjugated agarose beads; lane 3, immunoprecipitated HA-CKI using α -HA-conjugated agarose beads.

The *S. cerevisiae* CKI homologue, HRR25, is the principal yeast kinase that phosphorylates 14-3-3 at the site equivalent to residue 233

The cytosolic protein kinase from *S. cerevisiae* was partially purified by chromatography on an SP-Sepharose column. The kinase activity eluted from this cation exchange column at a similar molarity of NaCl (~ 0.4 – 0.5 M) as CKI α from mammalian brain [26], indicating that the yeast protein is also a kinase with a basic isoelectric point. The pI of HRR25 is 9.3 and the pI of CKI α is 9.47. The peak fraction from the SP-Sepharose column phosphorylated wild-type 6His-tagged BMH2, GST-BMH1, GST-BMH2 and 14-3-3 ζ (Fig. 5A). There was no significant phosphorylation of 14-3-3 ζ T233A or the double phosphorylation site mutant, 14-3-3 ζ S185A/T233A, which suggests that residue 233 is the single site of phosphorylation on mammalian 14-3-3 for CKI α .

To test this hypothesis, budding yeast cytosolic protein extracts were loaded onto SDS-PAGE minigels

and the gels overlaid with 6His-BMH1 wild-type and BMH1/S237A, the equivalent site to mammalian 14-3-3 S233. An in-gel kinase assay was then performed with [32 P]ATP/Mg $^{2+}$ and the gel was autoradiographed. 6His-BMH1 was phosphorylated by the cytosolic protein extract of wild-type yeast (Fig. 5B), whereas the BMH1/S237A mutant showed only weak phosphorylation. 6His-BMH1 was also incubated with a gel loaded with yeast extracts from a yeast strain containing an HRR25 deletion. This also resulted in weak phosphorylation of BMH1. These results indicate that HRR25 is the budding yeast kinase that is principally responsible for phosphorylation of BMH1 at Ser237.

The three other CKI homologues in *S. cerevisiae* (YCK1-3) are largely, if not totally, membrane-associated. Of the four CKI homologues in the *S. cerevisiae* genome (YCK1, YCK2, YCK3 and HRR25) [33,34], YCK1, 2 and 3 all have a very strong consensus sequence for prenylation and are membrane-associated, although some studies indicate that YCK3 may only

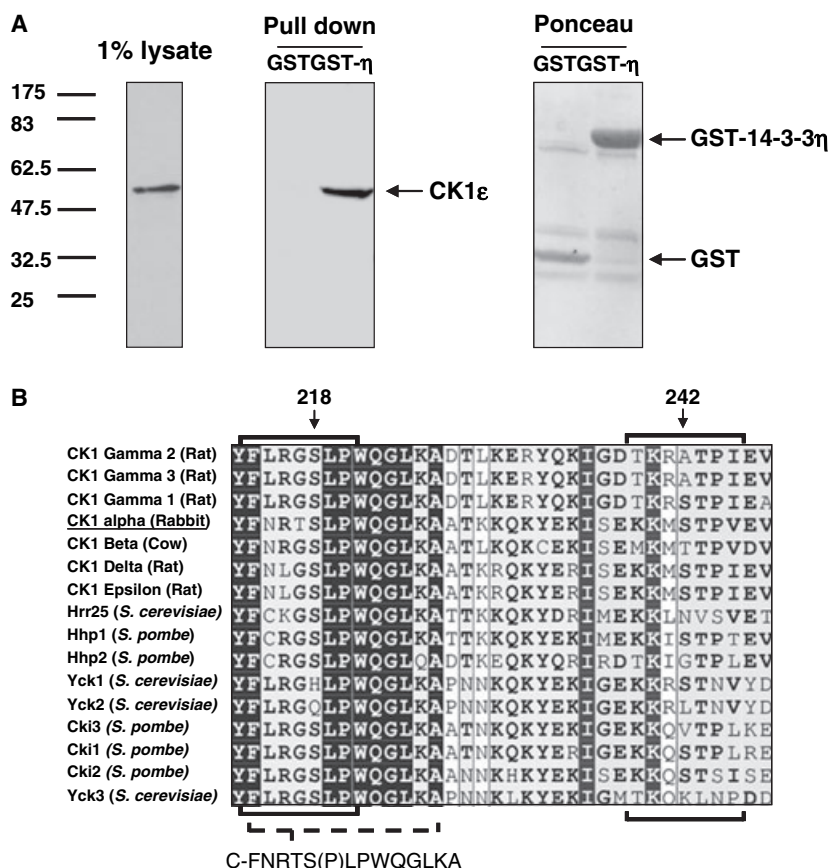


Fig. 4. 14-3-3 η binds to the CKI ϵ isoform *in vitro*. (A) HA-CKI ϵ was transfected into COS-7 cells, lysed and clarified by centrifugation. A sample of 1% of the COS-7 lysate transfected with HA-CKI ϵ is shown in the left hand panel. Equal amounts of GST or GST 14-3-3 η were incubated with the lysate for 2 h. GST or GST-14-3-3 η was recovered by glutathione beads and separated by SDS-PAGE. Western blotting with α -HA antibody revealed the presence of CKI ϵ in the GST-14-3-3 pull-down, but not the GST control (middle panel). Ponceau S staining revealed that similar amounts of GST and GST-14-3-3 η were incubated with the lysate shown in the right hand panel. The results are taken from two independent experiments. (B) Sequence alignment around the potential 14-3-3 binding region in CKI isoforms.

be partly membrane-associated [35]. Therefore, HRR25 is likely to be the only CKI homologue present in the yeast cytosolic extract.

In addition to HRR25, 6His-BMH1 can also be phosphorylated by mammalian CKI α and by the *Schizosaccharomyces pombe* homologue, Cki1 (Fig. 5C). However 6His-BMH1/S237A cannot be phosphorylated by these kinases, again suggesting that Ser237 is the site of phosphorylation on BMH1. A C-terminal BMH2 deletion construct was also prepared, where 40 residues were deleted from the C-terminus (BMH2 Δ 40). This construct lacked residue 233 (sequence TSDIS ... onwards), where the latter serine is the phosphorylatable residue. This construct was also only very weakly phosphorylated by HRR25 (data not shown), indicating that this region of the protein contained the HRR25 phosphorylation site.

To determine whether the HRR25 kinase could bind to the yeast BMH1, cytosolic extracts from *S. cerevisiae* were passed through a GST-BMH1 affinity column and, after extensive washing, the protein was eluted and incubated with BMH1 under kinase assay conditions, with GST used as a control

(Fig. 5D). This assay shows that both 6His and GST-BMH1 bind to HRR25 and that the control GST does not.

Activation of protein kinase A (PKA) increases association of 14-3-3 with CKI α in HEK 293 cells

The 14-3-3 binding motif R(S)X_{1,2}pSX(P) is generally a good consensus for a number of kinases, including PKA, Ca²⁺-calmodulin kinase II, protein kinase C (PKC) and AKT [36]. SCANSITE analysis (<http://scan-site.mit.edu>) of the CKI α sequence revealed a PKA or PKC phosphorylation site around the possible 14-3-3 binding motif at Ser242.

From an analysis of over 400 experimentally verified PKA sites in the Phospho.ELM database (<http://phospho.elm.eu.org/>), ~ 58% have two basic residues at expected positions; 35% have one; and 7% have no basic residue at position-3. It is clear therefore that many actual PKA substrates have a consensus similar to that found around the Ser218 site on CKI α (i.e. just one basic residue located near the amino terminus).

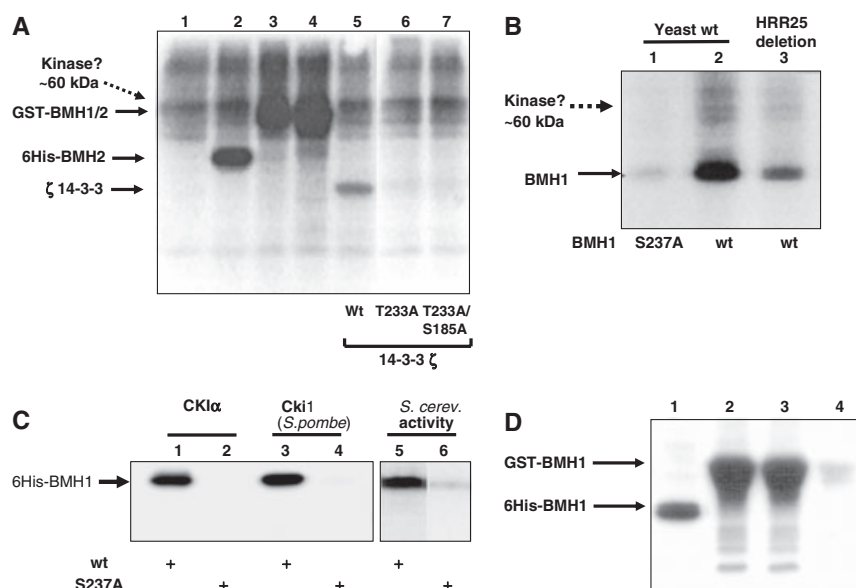


Fig. 5. Phosphorylation of mammalian and yeast 14-3-3 by yeast CKI homologues. (A) The peak fraction of kinase activity from the SP-Sepharose column was assayed (see Materials and methods) for its ability to phosphorylate the following constructs: lane 1, control (no added 14-3-3); lane 2, 6His-tagged BMH2 wild-type; lane 3, GST-BMH2; lane 4, GST-BMH1; lane 5, 14-3-3 ζ wild-type; lane 6, 14-3-3 ζ T233A; lane 7, 14-3-3 ζ S185A/T233A. The SP-sepharose purification was carried out on two separate occasions. The kinase activity, which eluted in three or four major fractions was shown to phosphorylate both the yeast 14-3-3 homologues (but not the S237A mutants; data not shown). On each occasion, one of the peak fractions was subsequently used to phosphorylate the constructs indicated in each lane. 14-3-3 ζ wild-type (lane 5) was assayed in duplicate in two separate lanes, one of which has been excised for clarity. (B) Cytosolic protein extracts from budding yeast were loaded onto SDS-PAGE minigels and were overlaid with 6His-BMH1 wild-type and BMH1/S237A. An in-gel kinase assay was then performed with [32 P]ATP/Mg $^{2+}$ and the gel was autoradiographed to identify whether active kinase is present. Lane 1, 6His-tagged BMH1, S237A; lane 2, 6His-tagged BMH1 wild-type; lane 3, 6His-tagged BMH1 phosphorylated by cytosolic protein extract from yeast HRR25 deletion mutant strain. This is a representative example of similar assays carried out on three separate occasions with similar results being obtained. (C) Left hand panel: purified His-tagged recombinant yeast 14-3-3, 6His-BMH1, wild-type (wt) and Ser>Ala mutant were phosphorylated by mammalian CKI α and by the *S. pombe* homologue, CKI1 (Millipore) using an *in vitro* kinase assay. Lanes 1 and 3, 6His-tagged BMH1 wild-type; lanes 2 and 4, 6His-tagged BMH1, S237A. This assay was performed in duplicate with similar results being obtained. Right hand panel: in-gel protein kinase assay of yeast cytosolic protein extract loaded on a number of lanes in a separate SDS-PAGE minigel, containing 6His-BMH1 wild-type and 6His-BMH1 S237A. The kinase assay was carried out with [32 P]ATP/Mg $^{2+}$ and autoradiographed. For clarity, only one lane per gel is shown. Lane 5, 6His-BMH1 wild-type; lane 6, 6His-BMH1/S237A. This assay is a control showing the specificity of the *S. cerevisiae* kinase for the S237 site. This has been demonstrated many times with both BMH1 and BMH2 GST- and 6His constructs. (D) An aliquot of the bound material was eluted from an affinity column of GST-BMH1 and an in-gel kinase assay was carried out. Lane 1, phosphorylation of 6His-BMH1; lanes 2 and 3, phosphorylation of GST-BMH1 (in duplicate); lane 4, kinase activity of protein eluted from control beads (from an affinity column of GST alone), assayed with GST-BMH1 as substrate. This is a representative example of binding assays carried out on two separate occasions with similar results being obtained.

The phosphatase inhibitor, NaF was added to CKI α expressed as a 35 S-labelled *in vitro*, transcription, translation (IVTT) product and binding assays were performed using GST-14-3-3 ζ and GST as a control. Binding was shown to increase on treatment with NaF, indicating a phospho-dependent binding mechanism. After incubation with NaF, two- to three-fold more CKI α associated with 14-3-3 than in a control incubation without NaF (Fig. 6A, compare lane 4 with 6). Densitometry was used to quantify the increase (Fig. 6C). A Coomassie Brilliant Blue stain on the right shows that similar amounts of GST and GST-14-3-3 were incubated with the IVTT reaction (Fig. 6B).

A similar experiment was carried out in which recombinant PKA was added to the assay after IVTT synthesis, along with NaF; however, no additional increase was seen (data not shown).

Because we had established that 14-3-3 η and 14-3-3 γ associated more strongly than other isoforms with CKI α in mammalian cells, for future binding experiments using cell culture, we focussed on the association of these endogenous 14-3-3 isoforms with CKI α . To determine whether PKA could stimulate (either directly or indirectly) phosphorylation of Ser218 on CKI α , and thus induce association with 14-3-3, HA-CKI α was transfected into HEK293 cells and PKA was activated

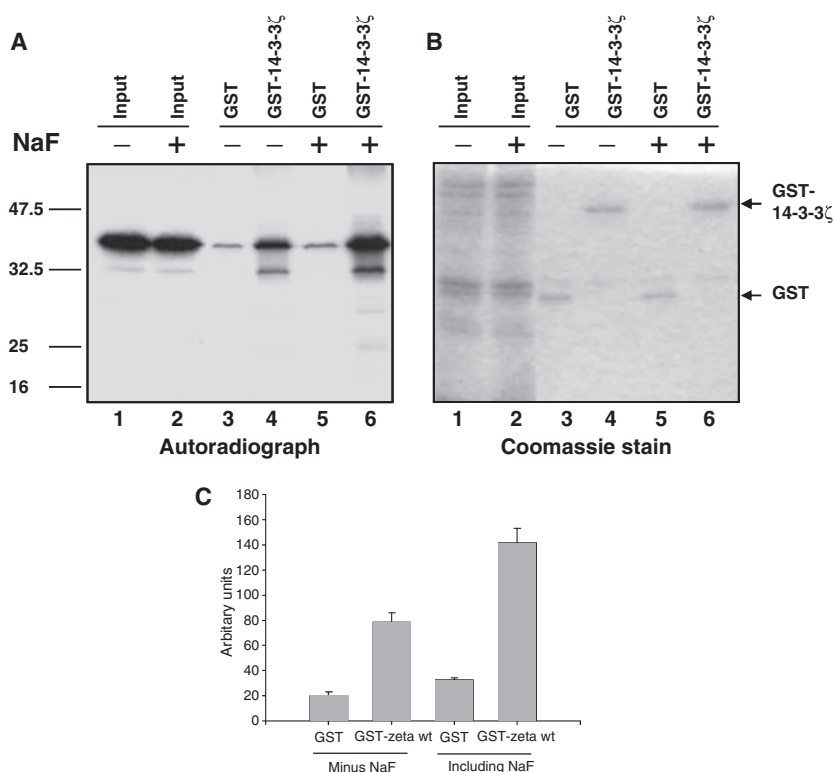


Fig. 6. 14-3-3 binds CKI α in a phosphorylation-dependent manner. (A) CKI α was produced by IVTT in the reticulocyte lysate (see Materials and methods) for 90 min, and then incubated with and without NaF for an additional 30 min at 30 °C before being tested for interaction with 14-3-3. Lanes 1 and 2, 1% of the lysate used for the untreated and phosphatase inhibitor treated (NaF) IVTT reactions, respectively; lanes 3 and 5, GST controls; lanes 4 and 6, GST-14-3-3 ζ association with CKI α . (B) Coomassie Brilliant Blue stain showing that equal amounts of GST and GST-14-3-3 were incubated with the IVTT reaction. (C) Densitometry of three independent experiments was used to quantify the increase in binding between the GST and GST-14-3-3 ζ with and without NaF treatment.

with the addition of dibutyl-cAMP (db-cAMP). A transient increase in association with 14-3-3 η was observed (Fig. 7A) after 10 min. Loading controls (Fig. 7B–D) indicate that equal amounts of 14-3-3 η and β -actin were present in the lysate and that equal amounts of CKI α were present in each immunoprecipitation. A repeat of this experiment with shorter time points (2 and 5 min) showed maximal binding at an even earlier time point of 5 min (data not shown). This time scale is consistent with previous studies examining PKA activation. Zhang *et al.* [37] were able to observe PKA activation by forskolin or db-cAMP in real time using fluorescence resonance energy transfer and a specially created construct containing 14-3-3 fused to a flexible loop region containing a perfect PKA phosphorylation site within a 14-3-3 binding motif. Binding of 14-3-3 to CKI α decreased, even below the level of original binding, after 60 min, possibly as a result of phosphatase activity and/or translocation of CKI α after 14-3-3 binding.

CKI α expressed by IVTT associates with 14-3-3 η in a phosphorylation-dependent manner

After observing that PKA activation by db-cAMP increased the association between 14-3-3 η and CKI α , intact wild-type CKI α was expressed by IVTT and

incubated with GST-14-3-3 η in the presence of db-cAMP. The phosphorylation state of CKI α within the reticulocyte lysate was also increased by incubating the lysate with phosphatase inhibitor. These results (Figs 7 and 8) suggest that a basal level of interaction is possible between 14-3-3 η and CKI α , which may be phosphorylation dependent. The interaction between CKI α and 14-3-3 η was not completely abolished by a site-directed mutant S218A (Fig. 8) and further IVTT analysis showed that constructs lacking residues 217–233 still showed some interaction with 14-3-3 η (data not shown). This finding is in contrast to the interaction of constructs containing this region with centaurin- α 1 [26]. We therefore searched in this region for other potential 14-3-3 binding motifs. Because the serine at 242 (KKMpS²⁴²TP) is a good consensus, we made the S242A mutation of this residue and a double S \rightarrow A mutant of both residues 218 and 242.

Figure 8 shows that the S218A mutation caused a significant reduction in 14-3-3 η binding compared to wild-type CKI α , whereas S242A and double S218/242A mutation reduced 14-3-3 η binding almost entirely. This experiment was repeated in COS-7 cells with similar results being obtained (data not shown). The S242A mutant showed almost complete loss of interaction, suggesting that, in these cell lines, the as yet unknown physiologically relevant kinase(s) were

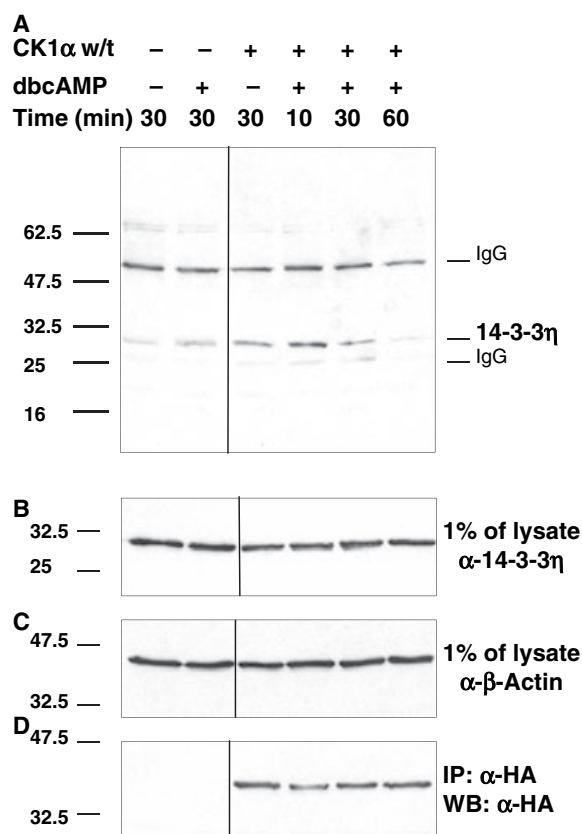


Fig. 7. Stimulation of PKA in 293 cells causes an increased association of endogenous 14-3-3 with CKI α wild-type. (A) HEK293 cells, transfected with CKI α , were serum starved for 18 h, and then stimulated with db-cAMP for the indicated times. The two left hand lanes are controls with no transfected CKI α , with and without db-cAMP. An immunoprecipitation was performed in the nontransfected cells using CKI α antibody to check that 14-3-3 η interacted endogenously with CKI α . The third lane shows unstimulated cells transfected with CKI α as a control; the next three lanes show an increasing time of incubation with db-cAMP. Stimulation of PKA for 10 min induced the greatest amount of 14-3-3:CKI α association; thereafter, the association diminished. (B, C) One percent of the lysate was western blotted with α -14-3-3 η and with α - β -actin. (D) The immunoprecipitated HA-CKI α blot was stripped and re-probed with α -HA after blotting with α -14-3-3 η (lower panels). These results are typical of three independent experiments.

relatively inactive and that the basal level of phosphorylation of Ser218 was low. Therefore, this indicates that the phosphorylation of S242 is more crucial for 14-3-3 η binding than S218.

Discussion

By contrast to a number of other brain proteins including centaurin- α 1, 14-3-3 did not co-purify with CKI α and we did not observe an association between

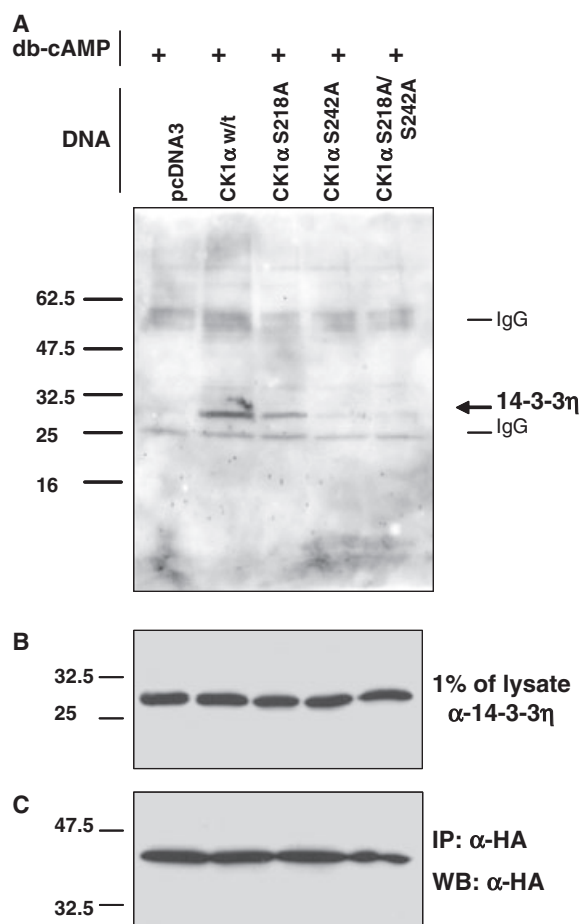


Fig. 8. Residues Ser218 and Ser242 of CKI α are required for 14-3-3 association. (A) Transfected HEK293 cells with point mutations of HA-CKI α were serum starved, and then stimulated with db-cAMP for 10 min. The cells were lysed and HA-CKI α immunoprecipitated with α -HA antibodies (clone HA-7 conjugated to agarose beads). The lysates were extensively washed and western blotted with anti-14-3-3 sera. The lanes from left to right show empty vector control; wild-type CKI α ; CKI α S218A; CKI α S242A; and CKI α S218A/S242A. (B, C) Control blots showing 14-3-3 levels. Equal amounts of CKI α in each immunoprecipitation are shown in the lower two panels. The blots are representative of three separate experiments.

14-3-3 and an affinity column comprising the unphosphorylated peptide corresponding to this region of CKI [25]. In the present study, we confirm that the interaction between 14-3-3 and CKI α is phosphorylation-dependent, with increased binding with the phosphorylated peptide. By contrast, centaurin- α 1, which is a phosphatidylinositol 3,4,5-trisphosphate binding protein involved in the modulation of vesicular trafficking and actin cytoskeleton organization, and comprising a GTPase-activating protein for ARF6 [38], binds only when the peptide has been dephosphorylated using PPase treatment.

14-3-3 η and γ isoforms bind most tightly to both the phospho-peptide and the dephosphopeptide and have been identified both *in vitro* and *in vivo* as binding most strongly to CKI α (Figs 1–3). The highly conserved nature of 14-3-3, in particular within the binding pocket, suggests that very subtle binding differences must exist to explain exactly how the same ligand can preferentially bind different 14-3-3 isoforms. The interaction of CKI α most probably occurs through contact with the basic pocket within 14-3-3 η , and is potentially further mediated through different contacts within the 14-3-3 dimer, perhaps aiding the observed isoform binding specificity. The crystal structure(s) of CKI have identified this region as being part of an unstructured loop that could be involved in protein interactions. Although unlikely, mutations to alanine (at positions 218 and 242) could have altered the local structure of CKI α in such a way as to decrease binding to 14-3-3, not just as a result of the removal of a phosphorylatable residue.

We have shown that Ser233 on 14-3-3 τ is the residue phosphorylated by BCR *in vitro* [39]. By contrast to CKI α , BCR phosphorylates the 14-3-3 τ isoform to a greater extent than 14-3-3 ζ . CKI ϵ also interacted with 14-3-3 η (Fig. 4). It may therefore be concluded that 14-3-3 interacts with other CKI isoforms if they contain a consensus 14-3-3 motif at the equivalent position of either residue 218 or 242. However, this region may well have a specific repertoire of binding molecules because recent studies found that this region in CKI δ could not interact with MAP1A [40], suggesting it is not the only interaction region within CKI.

The C-terminal regions of CKI δ [41] and CKI ϵ [42] can be hyperphosphorylated, causing autoinhibition of the isoforms, presumably by binding into or obscuring the active site such that it cannot access substrate. CKI ϵ contains an almost identical sequence around Ser218 compared to CKI α and a totally conserved sequence around Ser242. The fact that CKI ϵ binds 14-3-3 shows that the extended C-terminal in CKI ϵ does not interfere with binding. As noted earlier, this region is highly conserved throughout CKI isoforms; therefore, it is likely that other CKI isoforms will also interact through the region around Ser218.

There are many examples in the literature of 14-3-3 binding in an isoform-specific manner (e.g. Cbl, chloride intracellular channel 4, insulin-like growth factor-1, nuclear factor of activated T cells 3, PKC ζ and Par3 α), although the issue of isoform binding specificity is often not fully addressed in the literature. The data reported in the present study suggest that a binding preference exists for CKI α , and that the isoform 14-3-3 σ was unable to bind intact CKI α from cell

extracts. This 14-3-3 isoform shows some structural differences [43] and has a well-characterized specific role in the regulation of the cell cycle. The expression of 14-3-3 σ is induced after DNA damage by the transcription factor of tumour suppressor gene p53. 14-3-3 σ then arrests the cell cycle at the G₂/M checkpoint by sequestering Cdc2 into the cytoplasm [44]. Another example of a specific role for an isoform is provided by the zeta isoform, whose down-regulation has been shown to suppress anchorage-independent growth of lung cancer cells [45].

Addition of the phosphatase inhibitor NaF or modulation of PKA activity in HEK293 cells affected the amount of 14-3-3 association with CKI α , suggesting that the interaction can be regulated *in vivo*, even if not directly by PKA, and opens up possibilities for future studies into the regulation of CKI:14-3-3 association.

The interaction between 14-3-3 η and CKI α was not completely abolished by mutating Ser218 on CKI α and further analysis revealed that Ser242 is a binding site for 14-3-3. The results obtained from both cell transfection and immunoprecipitation studies indicate that CKI α is phosphorylated on both Ser218 and Ser242 and interacts in a phosphorylation-dependent manner with 14-3-3 isoforms. The CKI α mutant S218A had a reduced ability to associate with 14-3-3, whereas mutation of S242A reduced the binding almost completely. The double mutation completely abolished binding and had the same effect as the single S242A mutation; therefore, two possibilities are apparent. One is that Ser242 is the major site of the 14-3-3 phospho-dependent interaction and the other is that a S \rightarrow A mutation at this position changes the local structure or conformation of CKI in such a way as to decrease the binding affinity. This could be a result of the different binding affinities of 14-3-3 for these sites or different levels of kinase activity and/or kinase selectivity toward these sites.

A possible scenario could be that each 14-3-3 monomer of the 14-3-3 dimer could bind a phosphorylated residue of Ser218 and Ser242 simultaneously, after phosphorylation by PKA/PKC or another kinase. Such 'bidentate' binding has previously been observed for molecules such as Raf, BAD and Cbl [9,46].

A further possibility is that the Ser242 interaction is behaving like a 'gatekeeper', binding 14-3-3 first, and then allowing Ser218 (with presumably lower affinity) to bind into the other binding pocket of the 14-3-3 dimer, according to the 'gatekeeper hypothesis' [47]. This may help to explain the 14-3-3 isoform binding specificity because this region of CKI isoforms around Ser242 is slightly less conserved than around Ser218 (Fig. 4B).

Computer docking simulations were performed using ZDOCK software (<http://zdock.bu.edu/software.php>)

with respect to the known structures of a truncated CKI [30] and 14-3-3 [6,7], aiming to determine whether a dimeric 14-3-3 could bind CKI in a conformation where each subunit contacts a phospho-S218 and phospho-S242. The distances calculated between residues corresponding to Ser218 and the Ser242 peptide backbone and residues within the phosphate binding pocket of 14-3-3 suggested that these may be too great for phospho-S218 and phospho-S242 to bind in the phospho-binding pockets of the same 14-3-3 dimer (data not shown), and a fairly large structural movement might be required to accommodate simultaneous binding to CKI.

In conclusion, we have shown that CKI α interacts with 14-3-3 in a phosphorylation-dependent manner. This was demonstrated both *in vitro* and *in vivo* using HEK293 cells, Cos-1 cells, Cos-7 cells and in yeast as well as sheep brain (data not shown). However, the phosphorylation state of CKI α *in vivo* remains to be determined. We have previously shown that PKC isoforms phosphorylate centaurin- α 1 and reduce the association with CKI α [48]. Therefore, the difference in phosphorylation dependence of the interactions that we have demonstrated in the present study has important implications for the respective roles of centaurin- α and the 14-3-3 isoforms in the regulation of signalling through CKI isoforms.

Materials and methods

Materials

All chemicals and reagents were obtained from Sigma-Aldrich (St Louis, MO, USA), except for ATP (Redivue adenosine 5' [32 P]triphosphate[γ P], triethylammonium salt), which was obtained from GE Healthcare (Chalfont St Giles, UK). Pre-stained protein marker was obtained from New England Biolabs (Ipswich, MA, USA). Protease inhibitor tablets were obtained from Roche (Basel, Switzerland). The catalytic subunit of PKA was obtained from Merck (San Diego, CA, USA). The *S. pombe* CKI homologue, CKI1 (P40233) was obtained from Millipore (Billerica, MA, USA).

Molecular biology

The cDNAs for all 14-3-3 isoforms used for the peptide affinity experiments were obtained from various sources. 14-3-3 β (P39146), is an IMAGE clone (4843961/gi14060448) and was subcloned from the supplied vector (pOTB7) PCR with two oligonucleotides: 5'-GATC GAATTCATGACAATGGATAAAAGTGAGCTGGTA-3' and 3'-GATC GTCTCGACTTAGTTCTCTCCCTCCCCAG-5', creating an *Eco*R1 and a *Sal*I restriction site, respectively (underlined). The PCR product was inserted into pGEX-

4T1 (GE Healthcare), creating an N-terminal GST fusion. 14-3-3 η (Q04917), 14-3-3 γ (P61981) and 14-3-3 σ (P31947) were a gift from Henrik Leffers (University Department of Growth and Reproduction, Rigshospitalet, Copenhagen, Denmark), and the η and γ clones were present as an N-terminal GST-fusion in the vector pGEX-2TK (GE Healthcare). The 14-3-3 σ was subcloned from the vector pGPT-delta 6 using the oligonucleotides 5'-GATCGAAT TCATGGAGAGAGCCAGTCTGATC-3' and 3'-GATCGT CGACTCAGCTCTGGGGCTCCT-5' creating an *Eco*R1 site and a *Sal*I site, respectively (underlined). The PCR product was inserted into pGEX-4T1. 14-3-3 ζ (P63104) was obtained from a human cDNA library and was produced as an N-terminal GST fusion in the pGEX-2T vector. 14-3-3 ϵ (P62260) was produced as an N-terminal MBP fusion from a rat cDNA (accession m84416). Human 14-3-3 τ (P27348) was obtained from a previous study [49]. BMH1 (P29311) and BMH2 (P34730) were cloned as described previously [5]. CKI ϵ (P49674) was a gift from David Virshup (Johns Hopkins University School of Medicine, UH, USA) and was cloned into pS752. CKI α (P67828) was a gift from Peter Roach (Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN, USA) and centaurin- α (Q63629) was obtained from Anne Theibert (University of Alabama at Birmingham, Birmingham, AL, USA). All cDNAs were checked by sequencing both strands (Cytomyx, Cambridge, UK).

Recombinant protein purification

All GST-14-3-3 fusion cDNAs were transformed into *Escherichia coli* BL21 (DE3) pLysS competent cells (Merck), using the appropriate antibiotic. The cells were grown at 37 °C until a D_{600} of 0.9 was reached, then induced using isopropyl thio- β -D-galactoside from MP Biomedicals (Irvine, CA, USA) for 3.5 h at 30 °C, with shaking. The same procedure was used for the MBP-14-3-3 ϵ fusion but with the addition of glucose at 2 g·L $^{-1}$. Cell pellets, re-suspended in lysis buffer [NaCl/Pi, 1 mM phenylmethanesulfonyl fluoride, 1 mM EDTA, 1 mM dithiothreitol, protease inhibitor tablet (Roche) and 0.1% Triton] were lysed by sonication and clarified by centrifugation. The GST fusion protein was removed from the lysate using glutathione Sepharose 4B beads (GE Healthcare), and then the beads were washed extensively and the 14-3-3 cleaved off using thrombin (Sigma-Aldrich).

Immobilization of phosphopeptide

A synthetic peptide corresponding to residues 214–226 (CFNRTpSLPWQGLKA, where pS is phosphoserine) of CKI α was covalently attached to 'Sulpholink' gel (Pierce, Rockford, IL, USA) according to the manufacturer's instructions, through the cysteine residue, as introduced for this purpose, at the N-terminus. These were divided into

two equal amounts, with one aliquot being dephosphorylated by incubation with λ phosphatase and the other left phosphorylated. The beads were then incubated with HEK293 cell lysate for 2 h at 4 °C, with gentle rotation and, after extensive washing (five washes) in lysis buffer, the CKI-peptide 'pull-downs' were re-suspended in Laemmli buffer. Subsequently, four-fifths of each 'pull-down' was analysed by SDS-PAGE, followed by Coomassie Brilliant Blue staining and the remaining one-fifth was separated by SDS-PAGE followed by transfer for western blotting. The presence of 14-3-3 was confirmed by western blotting using anti-PAN 14-3-3 serum, which recognizes all 14-3-3 isoforms. Four percent of the lysate originally applied to the beads was loaded in a separate lane.

IVTT and pull-down assays

CKI α constructs were expressed *in vitro* using a T7 TNT coupled transcription/translation reticulocyte lysate (Promega, Madison, WI, USA). The 50 μ L reactions were performed in accordance with the manufacturer's instructions in a reaction mixture containing [35 S]methionine (GE Healthcare) for 90 min at 30 °C. Samples were made up to 200 μ L with binding buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 1% Nonidet-P40) and incubated for 15 min at 30 °C, with GST or GST-14-3-3 ζ . A further 300 μ L of binding buffer containing glutathione beads was added to the reactions and incubated at room temperature for an additional 1 h. The beads were washed five times with 1 mL of binding buffer and electrophoresed on 15% SDS-PAGE. After staining/destaining, the gels were incubated for 30 min with Amplify (GE Healthcare), dried and exposed to film.

Western blotting and antibodies

Western blot analysis was performed with the ECL detection system (GE Healthcare) using antibodies specific to each 14-3-3 isoform as described previously [32]. Western blots could be stripped and re-probed up to three times, after retesting the blots with the secondary antibody to ensure that the previous antibody had been removed. Antibodies to HA were obtained from Sigma-Aldrich (clone HA-7), anti-CKI α was from Santa Cruz (Santa Cruz, CA, USA) and anti- β -actin was from Millipore. Horseradish peroxidase coupled anti-rabbit (Bio-Rad, Hercules, CA, USA) and anti-mouse (Sigma-Aldrich) secondary sera were used. Samples were then analysed by SDS-PAGE, followed by autoradiography.

Yeast DNA manipulation

The yeast strain used was YPH252. DNA manipulations were performed in *E. coli* DH5a2. The BMH1 Ser>Ala 237 phosphorylation site mutant was generated by replacing

the wild-type sequence GAGATGTCCGAGT with GAGATGGCCGAGT and the BME1 Δ 40 deletion strain was produced as described previously [5]. The HRR25 (P29295) deletion strain was obtained from Brenda Andrews (University of Toronto, Canada) [50]

Extraction of protein from yeast

At least 5×10^8 cells per sample were harvested by centrifugation. The cell pellets were then frozen on dry ice in 1.5 mL Eppendorf tubes and could be stored at -70 °C. Further manipulations were carried out on ice or at 4 °C. To the cell pellet, 100 μ L of lysis buffer (25 mM Tris, pH 7.4, 100 mM NaCl, 10 mM EDTA, 1 mM dithiothreitol, 1% Triton X-100, 5% glycerol, 50 mM β -glycerophosphate, 20 mM NaF, 1 mM Na₃VO₄, 1 mM sodium pyrophosphate, with protease inhibitors; 1 mM phenylmethanesulfonyl fluoride and 1 mM each aprotinin, leupeptin, pepstatin A, chymostatin, 50 μ g·mL⁻¹ TLCK and 100 μ g·mL⁻¹ TPCK) were added. Sufficient acid-washed glass beads (0.5 mm diameter; Sigma-Aldrich) were added to fill up the depth of liquid and tubes were vortexed vigorously for 1 min. Fluid was removed from the beads, which were then washed once with another 100 μ L of lysis buffer. The lysis buffer extract was combined in a fresh tube and centrifuged in a microfuge for 3 min to remove insoluble material.

Purification of HRR25 kinase from yeast

The cytosolic protein kinase HRR25, from *S. cerevisiae* was partially purified by chromatography on an SP-Sepharose column as described previously [23].

Kinase assays

Twenty picomol of purified proteins were tested for their ability to be phosphorylated *in vitro* by CKI α as described previously [29] or by CKI δ (Millipore). Reactions were stopped by the addition of electrophoresis sample buffer and analysed on SDS/PAGE. Gels were stained with Coomassie Brilliant Blue and autoradiographed.

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sity School of Medicine, UH, USA). The HRR25 deletion strain was a kind gift from Dr Brenda Andrews (University of Toronto, Canada). We thank Marie Scarabel for preparation of some of the yeast constructs, including the BMH2 deletion construct. The CKI phosphopeptide was synthesized by Dr Cali Hyde (Wolfson Institute for Biomedical Research, UCL, London, UK). This work was supported by a Medical Research Council Programme Grant and a Parkinson's Disease Society, UK Project Grant (both to A.A.).

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